REFOLDING PROTEINS USING A CHEMICALLY CONTROLLED REDOX STATE

This application claims the benefit of U.S. Provisional Application No. 61/219,257 filed Jun. 22, 2009, which is incorporated by reference herein.

FIELD OF THE INVENTION

The present invention generally relates to refolding proteins at high concentrations, and more particularly to refolding proteins in volumes at concentrations of 2.0 g/L and above.

BACKGROUND OF THE INVENTION

Recombinant proteins can be expressed in a variety of expression systems, including non-mammalian cells, such as bacteria and yeast. A difficulty associated with the expression of recombinant proteins in prokaryotic cells, such as bacteria, is the precipitation of the expressed proteins in limited-solubility intracellular precipitates typically referred to as inclusion bodies. Inclusion bodies are formed as a result of the inability of a bacterial host cell to fold recombinant proteins properly at high levels of expression and as a consequence the proteins become insoluble. This is particularly true of prokaryotic expression of large, complex or protein sequences of eukaryotic origin. Formation of incorrectly folded recombinant proteins has, to an extent, limited the commercial utility of bacterial fermentation to produce recombinant large, complex proteins, at high levels of efficiency.

Since the advent of the recombinant expression of proteins at commercially viable levels in non-mammalian expression systems such as bacteria, various methods have been developed for obtaining correctly folded proteins from bacterial inclusion bodies. These methods generally follow the procedure of expressing the protein, which typically precipitates in inclusion bodies, lysing the cells, collecting the inclusion bodies and then solubilizing the inclusion bodies in a solubilization buffer comprising a denaturant or surfactant and optionally a reductant, which unfolds the proteins and disassembles the inclusion bodies into individual protein chains with little to no structure. Subsequently, the protein chains are diluted into or washed with a refolding buffer that supports renaturation to a biologically active form. When cysteine residues are present in the primary amino acid sequence of the protein, it is often necessary to accomplish the refolding in an environment which allows correct formation of disulfide bonds (e.g., a redox system).

Typical refold concentrations for complex molecules, such as molecules comprising two or more disulfides, are less than 2.0 g/L and more typically 0.01-0.5 g/L (Rudolph & Lilie, (1996) *FASEB J.* 10:49-56). Thus, refolding large masses of a complex protein, such as an antibody, peptibody or other Fc fusion protein, at industrial production scales poses significant limitations due to the large volumes required to refold proteins, at these typical product concentration, and is a common problem facing the industry. One factor that limits the refold concentration of these types of proteins is the formation of incorrectly paired disulfide bonds, which may in turn increase the propensity for those forms of the protein to aggregate. Due to the large volumes of material and large pool sizes involved when working with industrial scale protein production, significant time, and resources can be saved by eliminating or simplifying one or more steps in the process.

While protein refolding has previously been demonstrated at higher concentrations, the proteins that were refolded were either significantly smaller in molecular weight, less complex molecules containing only one or two disulfide bonds (see, e.g., Creighton, (1974) *J. Mol. Biol.* 87:563-577). Additionally, the refolding processes for such proteins employed detergent-based refolding chemistries (see, e.g., Stockel et al., (1997) *Eur J Biochem* 248:684-691) or utilized high pressure folding strategies (St John et al., (2001) *J. Biol. Chem.* 276(50):46856-63). More complex molecules, such as antibodies, peptibodies and other large proteins, are generally not amenable to detergent refold conditions and are typically refolded in chaotropic refold solutions. These more complex molecules often have greater than two disulfide bonds, often between 8 and 24 disulfide bonds, and can be multi-chain proteins that form homo- or hetero-dimers.

Until the present disclosure, these types of complex molecules could not be refolded at high concentrations, i.e., concentrations of 2.0 g/L and higher, with any meaningful degree of efficiency on a small scale, and notably not on an industrial scale. The disclosed methods, in contrast, can be performed at high concentrations on a small or large (e.g, industrial) scale to provide properly refolded complex proteins. The ability to refold proteins at high concentrations and at large scales can translate into not only enhanced efficiency of the refold operation itself, but also represents time and cost savings by eliminating the need for additional equipment and personnel. Accordingly, a method of refolding proteins present in high concentrations could translate into higher efficiencies and cost savings to a protein production process.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a series of plots depicting depicting the effect of thiol-pair ratio and redox buffer strength on product-species distribution; FIG. **1***a* depicts the effect of a 5 mM buffer strength; FIG. **1***b* depicts the effect of a 7.5 mM buffer strength; FIG. **1***c* depicts the effect of a 10 mM buffer strength; FIG. **1***d* depicts the effect of a 12.5 mM buffer strength; FIG. **1***e* depicts the effect of a 15 mM buffer strength and FIG. **1***f* depicts the effect of a 20 mM buffer strength.

FIG. **2** is a series of plots depicting the effect of the degree of aeration on the species distribution under fixed thiol-pair ratio and thiol-pair buffer strength.

FIG. **3** is an analytical overlay of a chemically controlled, non-aerobic refold performed at 6 g/L and optimized using an embodiment of the described method performed at 1 L and 2000 L.

SUMMARY OF THE INVENTION

A method of refolding a protein expressed in a non-mammalian expression system and present in a volume at a concentration of 2.0 g/L or greater comprising: (a) contacting the protein with a refold buffer comprising a redox component comprising a final thiol-pair ratio having a range of 0.001 to 100 and a redox buffer strength of 2 mM or greater and one or more of: (i) a denaturant; (ii) an aggregation suppressor; and (iii) a protein stabilizer; to form a refold mixture; (b) incubating the refold mixture; and (c) isolating the protein from the refold mixture.

In various embodiments the redox component has a final thiol-pair ratio greater than or equal to 0.001 but less than or equal to 100, for example within a range of 0.05 to 50, 0.1 to 50, 0.25 to 50, 0.5 to 50, 0.75 to 40, 1.0 to 50 or 1.5 to 50, 2 to 50, 5 to 50, 10 to 50, 15 to 50, 20 to 50, 30 to 50 or 40 to 50 and a Thiol-pair buffer strength equal to or greater than 2 mM, for